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Antioxidant Activity of Natural Astaxanthin Extracted from Shell of Arabian Red Shrimp *Aristeus alcocki* (Ramadan, 1938)

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Abstract

Shrimp processing waste is one of the important natural sources of carotenoid. The major component of carotenoids of shrimp and crab shell were mono and diesters of astaxanthin, a very potent antioxidant with some unique properties. Astaxanthin is a powerful quencher of singlet oxygen activity and a strong scavenger of oxygen free radicals. Antioxidant activity of shrimp shell astaxanthin was evaluated. The *in vitro* antioxidant activity of astaxanthin extract showed significant hydroxyl radical scavenging activity, superoxide anion scavenging activity and inhibition of lipid peroxidation. The IC_{50} values obtained were 56.43 ± 1.06, 27.91 ± 0.54 and 26.54 ± 0.42 ng ml^{-1}, respectively. Antioxidant activity of astaxanthin from *Aristeus alcocki* was obtained at nanogram levels. This powerful antioxidant function may be due to the unique molecular structure of astaxanthin and synergistic effect of astaxanthin and PUFAs present in the astaxanthin monoester and diester fractions.

Keywords: Astaxanthin, antioxidant property, shrimp shell waste, *Aristeus alcocki*

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Introduction

Oxidative stress has been proposed to play an important role in the progression of chronic diseases (Suwannalert et al., 2010). An imbalance between reactive oxygen and biological elimination system lead to oxidative stress (Favier, 2006; Buonocore & Groenendaal, 2007). The biologically relevant free radicals derived from oxygen are the superoxide anion (O_2^-), the per hydroxyl radical (protonated super oxide, HO_2), the hydroxyl radical ($^{\cdot}OH$), and free radical nitric oxide (NO). Some of these reactive molecules (e.g., superoxide, hydrogen peroxide and nitric oxide) are physiologically useful and, in fact, are necessary for life, but can also be harmful if present in excess or in inappropriate situations. Reactive oxygen species (ROS) become a problem when either a decrease in their removal or their overproduction occurs, resulting in oxidative stress (Athar, 2002). This stress and the resultant damage have been implicated in many diseases and a wealth of preventive drugs and treatments are currently being studied.

Carotenoids are very important biological antioxidants that act in both preventive and radical-scavenging roles. Carotenoids can also act as potent biological antioxidants, in mammalian and human cells mainly by two mechanisms; as quenchers of singlet oxygen and other reactive species, by absorbing the excited energy of singlet oxygen onto the carotenoid chain, leading to the degradation of the carotenoid molecule, but preventing other molecules or tissues from being damaged (Mortensen et al., 1997; Boileau et al., 1999; Beutner et al., 2001). Carotenoids can also act as chain-breaking antioxidants by scavenging free radicals thus preventing chain reaction that can lead to the degradation of unsaturated fatty acids, resulting in degradation of lipid-rich membranes. Astaxanthin's powerful antioxidant activity has been demonstrated in numerous studies showing detrimental effects of free radical-induced oxidative stress and its potential to target many important health conditions (Papas, 1999; Palozza & Krinsky, 1992a; Naguib, 2000). Astaxanthin, the most abundant carotenoid in the marine world has potential clinical applications due to higher antioxidant activity than other carotenoids (Miki, 1991; Kurashige et al., 1990; Tso & Lam, 1996). Astaxanthin is a carotenoid without pro vitamin A activity capable of crossing blood-brain barrier, hence the risk of hyper-vitaminosis and excessive accumulation of vitamin A is not a concern in exhibiting antioxidant properties (Tso & Lam, 1996). The present study is designed to study the antioxidant property of shrimp shell astaxantin in *in vitro* systems.

Materials and Methods

Shell waste of deep sea shrimp *Aristeus alcocki,* was collected from the processing plant, RF Exports Pvt. Ltd, Chandiroor and Caps Seafoods Pvt. Ltd, Vypeen, Kerala, India. The waste was transported to the laboratory in an insulated box in iced condition. They were packed in polyethylene bags and stored at -20^{0}C until use. The wet waste was homogenized in a laboratory mixer (Crompton Greaves, India) and used for the extraction of astaxanthin using acetone as per Barbosa et al. (1999). The extract was quantified by measuring absorbance at 470 nm using the equation of Kelley & Harmon (1972).

Acetone, potassium chloride, potassium hydroxide, petroleum ether (B.P. 40-60^{o}C) and n-butanol were purchased from Merck, India Ltd, Mumbai. Tris buffer, Tris-hydrochloride buffer, nitroblue tetrazolium (NBT), hydrogen peroxide, 2-deoxy-D-ribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium dodecyl sulphate (SDS), were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai. The other chemicals and reagents used were of analytical grade. Astaxanthin standard was purchased from Sigma chemicals, USA.

Analysis of different components in the shrimp shell extract was done using thin layer chromatography (TLC) of the extract based on the method of Kobayashi & Sakamoto (1999). The bands obtained were identified using standard astaxanthin and internationally accepted R_f values for astaxanthin monoester and astaxanthin diester. The astaxanthin monoester and diester fractions separated in TLC were saponified and the free fatty acids present were quantitatively converted to fatty acid methyl esters (FAME) using Boron triflouride-methanol reagent by the method of (Metcalfe et al., 1966). Methyl esters of fatty acids were taken in hexane and were separated by gas chromatography (Trace GC ultra, Thermo Electron Corporation) equipped with Elite 225 (Perkin Elmer) capillary column and a flame ionization detector in the presence of hydrogen and air. Nitrogen at a flow rate 0.5 ml min^{-1} was the carrier gas. Other GC conditions were: injector temperature – 250^{0}C; temperature programme- 110^{0}C- 4 min, 2.7^{0}C min^{-1} - 240^{0}C- 5 min; Detector at 275^{0}C. The fatty acids were identified and quantified by external standard method using the fatty acid standard mixture purchased from M/s. Suppleco. The output of the GC was integrated using Thermocad software (Thermo Electron Corporation, Italy) and individual fatty acids were expressed as per cent.

Antioxidant activity of the extract was studied by assaying superoxide anion scavenging activity, inhibition of lipid peroxidation and hydroxyl radical scavenging activity. Superoxide anion ($O_2^{\cdot -}$) generated from photo reduction of riboflavin was detected by nitroblue tetrazolium (NBT) reduction method of McCord and Fridovich, (1969). Quercetin was used as standard. Lipid peroxidation induced by Fe^{2+} - ascorbate system in beef liver homogenate was estimated by thiobarbituric acid reaction method of Ohkawa et al., (1979). Catechin was used as the standard. Hydroxyl free radicals degrades 2-deoxy ribose to form thiobarbituric acid reactive substances (TBARS) (Elizabeth & Rao, 1990). Hydroxyl radical scavenging activity was determined by studying the competition between deoxyribose and the astaxanthin extract for the hydroxyl radical generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction). Catechin was used as standard. The statistical analyses were done using the statistical package SPSS 15 for windows. Results are expressed as mean ± SD.

Results and Discussion

TLC analysis of the shell waste extract showed that it contained free astaxanthin, astaxanthin monoester and astaxanthin diester in the ratio 1:1:2. GC identification of the fatty acids esterified with astaxanthin revealed that saturated fatty acids, mono unsaturated fatty acids (MUFA) and poly unsaturated fatty acids (PUFA) were in the ratio 5:3:2 in monoester, whereas in diester they are in the ratio 4:3:3 (Table 1). The main fatty acids in monoester and diesters were palmitic acid, oleic acid, stearic acid and PUFAs: docosa hexaenoic acid (DHA) and eicosa pentaenoic acid (EPA). TLC of the extract showed three distinct bands of astaxanthin, astaxanthin monoester and astaxanthin diester. R_f values obtained were 0.33, 0.60 and 0.78, respectively. The R_f values obtained for astaxanthin monoester and astaxanthin diester are in agreement with the results reported by Kobayashi & Sakamoto (1999). The R_f value of astaxanthin obtained in the present study is in accordance with the R_f value obtained for the standard astaxanthin and also the R_f value reported by Todd (1998). The fatty acid profiles are in agreement with the findings of Maoka & Akimoto (2008), who reported that saturated fatty acids, MUFAs and PUFAs were present in the astaxanthin monoester and diester fractions of spiny lobster *Panulirus japonicus.*

Table 1. Fatty acid composition of astaxanthin monoester and astaxanthin diester, as % of fatty acid

Component	Area %	
	Astaxanthin monoester	Astaxanthin diester
C1	3.002	0
C14	4.161	2.121
C16	18.382	20.472
C16:1	2.930	4.249
C17	0.910	0.947
C18	9.281	8.800
C18:1	14.400	18.197
C18:2	1.236	2.232
C18:3	1.867	1.116
C20:1	8.468	6.333
C20:4	2.844	3.988
C20:3 & C21	2.922	1.690
C20:5	4.832	8.797
C22	7.388	3.201
C22:1	1.758	0.665
C22:6	6.577	11.360
C24	6.165	4.708
C24:1	2.878	1.125

Astaxanthin extract from shrimp shell waste possessed significant hydroxyl radical scavenging activity, superoxide anion scavenging activity and lipid peroxidation inhibiting activity (Table 2). The standard antioxidants quercetin and catechin showed antioxidant activity at microgram levels whereas astaxanthin present in shrimp shell extract showed *in vitro* antioxidant activity at nanogram levels. This clearly indicates the high antioxidant potential of astaxanthin from *Aristeus alcocki* shell waste.

This powerful antioxidant function may be due to unique molecular structure of astaxanthin. Natural compounds are excellent singlet oxygen quenchers as well as lipid peroxidation chain breakers. Astaxanthin is such a natural compound whose dual antioxidant capacity may be attributed to the activity of polyene chain. Higher the number of conjugated double bonds along the polyene chain, higher will be the antioxidant activity. Cardounel et al. (2003) reports that such compounds can entrap free radicals, neutralize singlet and triplet oxygen. Wang et al. (1993) report that carotenoids can take part in protecting against the damage from free radicals and singlet oxygen reactive species. Sewer et al. (1998) showed that astaxanthin has a strong quenching capability against damage from singlet oxygen *in vitro.* This quenching capability has been shown to be 80 times stronger than α-tocopherol and twice as strong as α-carotene. Sewer et al. (1998) argued that the strong singlet oxygen quenching capability of astaxanthin is due to to its unique molecular structure, by virtue of which singlet oxygen associated carbon centered radicals of astaxanthin can form more stable resonance structures by the attachment of carbonyl groups and hydroxyl groups to the α-ionone ring of astaxanthin.

Palozza & Krinsky (1992b) have reported that astaxanthin can remove lipid peroxyl radicals in the liposomal suspension more efficiently than α-carotene but less efficiently than α-tocopherol. This is due to the fact that hydrogen bonding of the carbonyl group in the α-ionone ring of astaxanthin and hydrophobic association of the polyene chain allow AST to exhibit strong antioxidant effect both *in vitro* and *in vivo.*

Feeding study with salmon (Bell et al., 2000) showed that the antioxidant synergism of vitamin E and astaxanthin reduced malondialdehyde formation in an *in vitro* stimulation of microsomal lipid peroxidation. Oxygen derived free radicals or ROS formed in the body during energy producing metabolic process, play an important role in pathophysiology of a number of diseases (Cuzzocrea et al., 2001). Normally oxygen free radicals are neutralised by natural antioxidants. However, ROS become a problem when either a decrease in their removal or their overproduction occurs, resulting in oxidative stress. This stress and the resultant damage have been implicated in many diseases and a wealth of preventive drugs and treatments are currently being studied. Thus astaxanthin,

Table 2. *In vitro* antioxidant activity of astaxanthin from shrimp shell waste (*Aristeus alcocki*), IC_{50}

Activity	Astaxanthin (Mean ± SD)	Quercetin (Mean ± SD)	Catechin Mean ± SD)
Superoxide radical scavenging activity	27.91 ± 0.54 ng ml^{-1}	41.21 ± 0.76 µg/ml	ND
Inhibition of lipid peroxidation	26.54 ± 0.42 ng ml^{-1}	ND	432 ± 10.2 µg ml^{-1}
Hydroxyl radical scavenging activity	56.43 ± 1.06 ng ml^{-1}	ND	842 ± 16 µg ml^{-1}

n=6 ND = Not Detected

exhibiting multiple antioxidant activity will find utility in applications like antioxidant therapy, which is based on reducing oxidative stress in the target tissues. Since synthetic astaxanthin is a mixture of all types of isomers astaxanthin from natural sources is preferred as an antioxidant. Astaxanthin from natural sources is abundant in the isomer showing highest biological activity (3R, 3′R; 3S 3′S).

Singlet oxygen quenching ability of astaxanthin esters from the green algae *Haematococcus pluvialis* has been reported by Kobayashi & Sakamoto (1999). They showed that astaxanthin esters function as powerful antioxidant agents under both hydrophilic and hydrophobic conditions. The IC_{50} values of astaxanthin diester, astaxanthin monoester and free astaxanthin from *Haematococcus pluvialis* for singlet oxygen quenching activity in 50% (v/v) hexane in ethanol were 7.4 µg ml^{-1}, 8.6 µg ml^{-1} and 9.4 µg ml^{-1} respectively. Kamath et al. (2008) have reported that the IC_{50} values for free radical scavenging activity of *Haematococcus pluvialis* astaxanthin esters *in vitro* were 8.0 µg ml^{-1}. In the present study IC_{50} values reported for *in vitro* antioxidant activity of astaxanthin from *Aristeus alcocki* shell waste are in the range of ng ml^{-1}. This clearly indicates that the astaxanthin extract from *Aristeus alcocki* is a more powerful antioxidant agent than the astaxanthin present in the *Haematococcus pluvialis.* This may be due to a higher proportion of astaxanthin diester and a higher content of poly unsaturated fatty acids (20% PUFAs in monoester and 30% PUFAs in diester) in the carotenoid extract obtained from *Aristeus alcocki* shell. Thus, the powerful antioxidant property of carotenoid extract of *Aristeus alcocki* may be attributed to the antioxidant synergism of astaxanthin and PUFAs present in the extract.

Present study reveals that shrimp shell astaxanthin is more powerful than algal astaxanthin and can find application in natural medicine. Even though natural astaxanthin from *Haematococcus pluvialis* is a key ingredient in many nutraceuticals and functional foods, astaxanthin from shrimp waste can be a more economical and environment friendly approach.

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